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Abstract: Armillaria mellea is a serious pathogen of horticultural and agricultural systems in Europe and North America, responsible for extensive crop losses and the majority of incidents of Armillaria root disease in UK gardens. The lack of a reliable in vitro fruiting system for heterothallic A. mellea has hindered research and necessitated dependence on intermittently available wild-collected basidiospores of endemic, unknown genotypes, resulting in the use of variable genetic material in transformation studies. Here we describe a reliable, reproducible fruiting method to produce mature basidiocarps with viable basidiospores from in vitro cultures of heterothallic A. mellea from the western US. We evaluated isolates, media and light and temperature conditions to determine the most effective conditions for in vitro fruiting body production. Following colonisation of rice, sawdust and tomato medium at room temperature for four weeks, cultures were incubated in growth rooms under warm / bright conditions for four to six weeks before being placed in dim / cool conditions to provide a light and temperature reduction. Primordia emerged within three to four weeks following a temperature decrease of at least 8°C and this was most efficient when coupled with a light reduction. Basidiocarps matured within three to four weeks and produced viable basidiospores. Strains of Agrobacterium tumefaciens and transformation vectors containing the hygromycin resistance gene (hph) were constructed and evaluated by transformation of in vitro-produced basidiospores. A versatile transformation vector, producing transformation efficiencies similar to the archetypal basidiomycete vector pBGgHg, was constructed to simplify promoter and marker gene exchange using the yeast homologous recombination system to facilitate future genetic work in this species. In addition, fruiting was induced in transgenic isolates and mycelial cultures established from transgenic fruiting bodies maintained hygromycin resistance. Fruiting bodies and viable basidiospores of A. mellea have been reliably produced in vitro which, coupled with the enhanced knowledge of suitable A. tumefaciens strains and vectors for transformation, will assist future genetic research into this important pathogenic species.

A reliable *in vitro* fruiting system for *Armillaria mellea* for evaluation of *Agrobacterium tumefaciens* transformation vectors

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Abstract

Armillaria mellea is a serious pathogen of horticultural and agricultural systems in Europe and North America, responsible for extensive crop losses and the majority of incidents of Armillaria root disease in UK gardens. The lack of a reliable *in vitro* fruiting system for heterothallic *A. mellea* has hindered research and necessitated dependence on intermittently available wild-collected basidiospores of endemic, unknown genotypes, resulting in the use of variable genetic material in transformation studies. Here we describe a reliable, reproducible fruiting method to produce mature basidiocarps with viable basidiospores from *in vitro* cultures of heterothallic *A. mellea* from the western US. We evaluated isolates, media, light and temperature conditions to determine the most effective conditions for *in vitro* fruiting body production. Following colonisation of rice, sawdust and tomato medium at room temperature for four weeks, cultures were incubated in growth rooms under warm / bright conditions for four to six weeks before incubation in dim / cool conditions to provide a light and temperature reduction. Primordia emerged within three to four weeks following a temperature decrease of at least 8°C and this was most efficient when coupled with a light reduction. Basidiocarps matured within three to four weeks and produced viable basidiospores. Different strains of

Agrobacterium tumefaciens and various transformation vectors containing the hygromycin resistance gene (*hph*) were constructed and evaluated by transformation of *in vitro*-produced basidiospores. A versatile transformation vector, producing transformation efficiencies similar to the archetypal basidiomycete vector pBGgHg, was constructed to simplify promoter and marker gene exchange using homologous recombination in yeast to facilitate future genetic work in this species. In addition, fruiting was induced in transgenic isolates and mycelial cultures established from transgenic fruiting bodies maintained hygromycin resistance. Fruiting bodies and viable basidiospores of *A. mellea* have been reliably produced *in vitro* which, coupled with the enhanced knowledge of suitable *A. tumefaciens* strains and vectors for transformation, will assist future genetic research into this important pathogenic species.

1. Introduction

Armillaria is a significant pathogen of agricultural and horticultural systems globally, causing Armillaria root disease on a wide range of species, primarily woody perennials. There are approximately 40 described species, one of which is *A. mellea* (Baumgartner *et al.* 2011). Once considered to be a polymorphic species of variable virulence present throughout the world, *A. mellea* was revealed to be a species complex, with 10 distinct species occurring across North America (Anderson & Ullrich, 1979), including *A. mellea sensu stricto. Armillaria mellea sensu stricto* is also present in Europe (Korhonen, 1978), as well as in Africa and Asia. Populations across Europe and North America are heterothallic, whereas those of Japan (*A. mellea* ssp. *nipponica*) (Cha & Igarashi, 1995) and Africa (*A. mellea* ssp. *africana*) are homothallic (Kile *et al.*1994), excluding recent introductions (Coetzee *et al.* 2001). China is the only place where both heterothallic (Chinese Biological Species K) and homothallic (CBS G) are known (Qin *et al.* 2007).

Armillaria mellea is one of the most virulent *Armillaria* species in the Northern hemisphere, responsible for annual yield losses of 10–40% in infected vineyards in California (Baumgartner, 2004; Morrison, 2004) and identified as the primary cause of Armillaria root disease in 79% of *Armillaria* infections assessed in UK gardens by the RHS (RHS, 2015). The genome of *A. mellea* has recently been sequenced (Collins *et al.* 2013) and is available online (genome.jgi.doe.gov/Armme1_1/Armme1_1.home.html).

Problematic *in vitro* fruiting of *Armillaria* has been emphasised in the literature (Anderson, 1982; Korhonen & Hintikka, 1974; Reaves & McWilliams, 1991; Rhoads, 1925; Rhoads 1945; Shaw *et al.* 1981). Indeed, the few successful reports of fruiting in *A. mellea sensu stricto* are mainly concerning homothallic subspecies from Japan (*A. mellea* spp. *nipponica*) (Ota *et al.*1998), Korea (likely to be *A. mellea* spp. *nipponica*) (Shim *et al.* 2006) and Africa (*A. mellea* spp. *africana*) (Abomo-Ndongo *et al.* 1997).

The ability to fruit *A. mellea* in culture ensures the regular supply of basidiospores for experimentation, where previously researchers had to rely on wild-harvested basidiospores from fruiting bodies of a local genotype that were only available periodically. Moreover, *A. mellea* is edible,

so there is some interest in deliberate cultivation. In this paper we describe a reliable, reproducible *in vitro* fruiting system for heterothallic *A. mellea* from the western US, and demonstrate the importance of a light and temperature reduction for primordia production, in accordance with fruiting requirements in other basidiomycetes (Kües & Liu, 2000). *Agrobacterium tumefaciens*-mediated transformation of *A. mellea* relies on the use of basidiospores (Baumgartner *et al.* 2010) and here we also evaluate the efficacy of *A. tumefaciens* strains and vectors, known to have varying efficiencies in other basidiomycetes (Burns *et al.* 2006; Collins *et al.* 2010; Heneghan *et al.* 2009; Kilaru *et al.* 2006; Kilaru *et al.* 2009), to explore their potential for use in future transformation studies.

2. Methods

2.1 Strains and culture conditions

Isolates of *A. mellea* (heterothallic and homothallic) and *A. gallica* were obtained from collections held at the Royal Horticultural Society, Wisley, UK and the United States Department of Agriculture-Agricultural Research Service, Davis, USA (Table 1). Wild-type isolates were originally identified by pairing with haploid testers (Guillaumin *et al.* 1991) or amplification of the IGS1 region and subsequent digestion with *Alu*I (Harrington & Wingfield, 1995). Isolates were routinely maintained on potato dextrose agar (PDA) at 25°C in the dark. *Saccharomyces cerevisiae* strain Y10000 was used for plasmid construction by homologous recombination and was maintained on yeast peptone dextrose agar (YPDA) at 28°C. *Escherichia coli* strain DH5α was used for subcloning plasmids and *Agrobacterium tumefaciens* strains AGL-1 and LBA1126 were used in *A. tumefaciens*-mediated transformation of *A. mellea*.

2.2 Fruiting media and growth room conditions

Several different media were evaluated to induce fruiting bodies. Media were prepared freshly on the day of use in 1 litre wide mouth jars (VWR) and the sawdust was a softwood pet bedding. The following media were utilised: Orange medium: two oranges (roughly chopped) and 500 ml water (Reaves & McWilliams, 1991); Rice, sawdust and carrot (RSC): 30 g rice, 15 g sawdust, 150 ml water and 1 cm top layer of homogenised carrot (Shim *et al.* 2006): Rice, sawdust and peptone (RSP): 30 g rice, 15 g sawdust and 150 ml of 0.6% peptone solution (Grillo *et al.* 2000); Rice, sawdust and tomato (RST): 30 g rice, 15 g sawdust, 150 ml water and 1 cm top layer of homogenised tomato; Rice, sawdust, pearl barley, corn and carrot (RSBCC): 20 g rice, 10 g sawdust, 10 g pearl barley, 10 g corn kernels, 150 ml water and 1 cm top layer of homogenised carrot. Media were inoculated with mycelial agar plugs from a four-week *Armillaria* culture and incubated at room temperature in the dark for four weeks, or until the mycelium had fully colonised the medium. After colonisation, the cultures were moved into their respective growth rooms for incubation with 70% relative humidity and photoperiods, temperatures and light intensities appropriate to the required experimental conditions. The growth

rooms were fitted with fluorescent lights with adjustable light intensities and cultures were wrapped in aluminium foil when complete darkness was required. Cultures were examined weekly for the presence of primordia.

Following production of fruiting bodies, basidiocarps were harvested and placed gills down on a clean black piece of paper, covered with a plastic tub and left for 24 hours in a cool room to obtain a spore print. The basidiospores were then scraped off the paper using a sterile wire loop, suspended in sterile deionised water and stored at 4°C until use. Basidiospores remained viable and transformable for at least two years post harvesting.

2.3 Vector construction and *Agrobacterium tumefaciens* transformation

Plasmid pBGgHg (Chen *et al.* 2000) is based on pCAMBIA and contains the full length hygromycin resistance gene, *hph*, under the control of the *Agaricus bisporus gpdII* promoter with the CaMV 35S terminator. Plasmid pGR4-4iGM3 (Burns *et al.* 2005), is based on pGreen and contains the truncated *hph* gene (where the first two lysine residues of the coding region are deleted) driven by the *A. bisporus gpdII* promoter with the *Aspergillus nidulans trpC* terminator.

Yeast homologous recombination using the LiOAc transformation method (Gietz & Woods, 2002) was used to adapt the *A. tumefaciens* binary vector pCAMBIA0380 with the 2 μ origin of replication and the *URA3* gene for plasmid maintenance and selection in a *ura3* mutant *S. cerevisiae* strain for use as a backbone vector (pCAMBIA0380YA) (Ali, 2015) in the construction of plasmids pCAM-hph-Pcgpd and pCAM-hph-series (Supplementary Figure 1). pCAM-hph-Pcgpd (containing the *Phanerochaete chrysosporium gpd* promoter, truncated *hph* gene and *A. nidulans trpC* terminator) and pCAM-hph-series (containing the *A. bisporus gpd* promoter, full length *hph* gene and CaMV 35S terminator) were designed in Clone Manager (Sci-Ed Software). The backbone vector pCAMBIA0380YA was linearised by digestion with *Bam*HI and PCR was performed using Phusion DNA polymerase (Thermo Scientific) and primers listed in Supplementary Table 1 to amplify the required DNA fragments from other plasmids for use in yeast homologous recombination to construct the vectors. Primers were designed to include a 30 bp overlap between the PCR fragments to allow recombination in yeast. Phusion PCR was performed at 98°C for 30 seconds, followed by 36 cycles of 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 45 seconds with a final cycle of 72°C for 10 minutes.

Plasmids were extracted from *S. cerevisiae* cells using Zymoprep Yeast Plasmid Miniprep II (Zymo Research), rescued into competent *E. coli* and verified by PCR using LB/RB primers (Supplementary Table 1), restriction digestion and sequence analysis. Correctly constructed plasmids were transformed into competent *A. tumefaciens* by electroporation.

Agrobacterium tumefaciens-mediated transformation of *A. mellea* was performed as per Baumgartner *et al.* (2010) using strains AGL-1 (Lazo *et al.* 1991) and LBA1126 (Bundock *et al.* 1995) carrying the four plasmids (Table 2). Putative transformants were subcultured on to PDA plates containing 200 μg/ml timentin and 30 μg/ml hygromycin. One-way analysis of variance (ANOVA) was performed in

SPSS to determine the effects of *A. tumefaciens* strain (AGL-1, LBA1126), vector (pBGgHg, pGR4-4iGM3, pCAM-hph-Pcgpd, pCAM-hph-series), and their combination on the efficiency of transformation. Means were compared for significant main effects ($P \geq 0.05$) using Tukey's HSD test in SPSS.

2.4 Confirmation of transgene presence in putative transformants

DNA was extracted from two-week PDB cultures using the protocol described by Liu *et al.* (2000) and PCR was performed using *hph* primers (Supplementary Table 1) to confirm presence of the transgene. The cycle used was 2 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 60 seconds at 72°C with a final cycle of 72°C for 10 minutes.

3. Results

3.1 Preliminary production of fruiting bodies

A variety of media and isolate combinations were used in the preliminary experiments in order to induce fruiting in isolates of *A. gallica* and *A. mellea* present in our collection (Table 3). All isolates were able to colonise all media after four weeks in darkness at room temperature (20 to 25°C). Following transfer into growth rooms with a photoperiod of 12 h light / 12 h dark and temperature 15°C, primordia were induced in cultures of *A. gallica* isolate CG645 and heterothallic *A. mellea* isolates CG440, ELDO17 and ELDO19 and these primordia developed into immature fruiting bodies in CG440 and ELDO19 but did not develop further. ELDO17 produced immature fruiting bodies on RSC, RST and RSBCC and mature fruiting bodies were obtained from the RST medium (Figure 1). All other isolates that produced primordia could do so on the RST medium. Primordia production appeared to be dependent upon a reduction in light intensity, photoperiod or temperature.

3.2 Establishment of an effective medium and light and temperature conditions

In order to establish an effective *in vitro* fruiting system for heterothallic *A. mellea*, experiments proceeded with heterothallic isolates ELDO17 and ELDO19 collected from the western US. In the preliminary experiments, primordia were only produced following a combined light and temperature reduction; therefore, an experiment was devised to determine whether this was a requirement for primordia induction. Three media were compared: RST, RSC and RSP, and cultures underwent a light and temperature reduction from 23°C to 15°C, photoperiod of 16 h light / 8 h dark to 10 h light / 14 h dark and light intensity from 125 to 5 µmol m^{-2} s^{-1}. Negative controls were cultures that were maintained in constant conditions of 15°C and 5 µmol m^{-2} s^{-1} light intensity with a photoperiod of 10 h light / 14 h dark. The experiments were repeated at least twice with five cultures of each isolate in total as a minimum. ELDO17 produced primordia on all three media tested and on RST most frequently (96%), whereas for ELDO19, primordia were produced on RSC (56%) and RST (79%), but not on RSP (Figure 2). No primordia developed in the control cultures, which did not undergo a light

and temperature reduction. Cultures usually produced several hundred primordia per pot and five to thirty of these progressed to immature fruiting bodies, with between one and five of these reaching maturity. Primordia of ELDO17 developed into mature fruiting bodies more regularly on RST (75%) than on RSC (22%), and not at all on RSP. ELDO19 only developed mature fruiting bodies on RSC, and very infrequently (11%) (Figure 2). Mature fruiting bodies usually developed within three weeks and basidiospores were viable and could be collected from all mature fruiting bodies providing that the caps were fully expanded and the spore print was taken in cool conditions (15 to 20°C). Monosporous progeny of ELDO17 and ELDO19 exhibited a white fluffy morphology indicative of a haploid culture.

3.3 Uncoupling of light and temperature requirements for ELDO17 fruiting body production

Further experiments were conducted to separate the effects of light and temperature on the development of fruiting bodies. In one experiment, initial colonisation temperature (18°C, RT or 25°C) had no effect on the timing or incidence of primordia production, but primordia did not develop into mature fruiting bodies if initially colonised at 18°C. Colonisation in the light reduced the proliferation of rhizomorphs in comparison to colonisation in darkness but did not affect fruiting body production. Variation of photoperiod in the warm / bright growth room from 8 to 16 hours of light and variation in temperature from 23°C to 28°C had no effect on the production of fruiting bodies, but at least four weeks' incubation in warm / bright conditions before decreasing the light and temperature was essential for primordia initiation.

To determine whether reduction in light or a reduction in temperature was responsible for primordia induction, RST medium was colonised with ELDO17 for four weeks (at RT unless specified) and the cultures were then incubated in thirteen conditions for six weeks (Table 4), before being incubated in an additional condition if required, awaiting emergence of primordia (Figure 3). This experiment was performed with three to six replicates for the various conditions.

All positive control cultures (condition 6) with the optimal parameters of warm / bright conditions of 23ºC, 125 µmol m^{-2} s^{-1} light for six weeks followed by cool / dim conditions of 15ºC, 5 µmol m^{-2} s^{-1} light produced mature fruiting bodies. No primordia were produced in cultures maintained in constant conditions of 15ºC or 23ºC and 5 µmol m^{-2} s^{-1} light, 125 µmol m^{-2} s^{-1} light (conditions 1, 2, 11, 12) or darkness (conditions 3 and 13) that did not experience a light or temperature reduction, nor in cultures maintained in darkness with a temperature reduction (condition 8). A temperature decrease was crucial for primordia initiation, and a light reduction was also important. At 23ºC a light reduction from 125 µmol m^{-2} s^{-1} to 5 µmol m^{-2} s^{-1} was unable to induce fruiting (condition 4), whereas at 15ºC a light reduction was sufficient for fruiting induction if colonisation occurred initially at RT (condition 9) (67% of cultures fruited) but not at 15ºC (condition 10). A temperature reduction alone from 23ºC to 15ºC was sufficient to induce primordia at 125 µmol m^{-2} s^{-1} (condition 7) (33% of cultures fruited) and 5 µmol m^{-2} s^{-1} (condition 5) (50% of cultures fruited).

3.4 Fruiting body production by homothallic isolates

Following successful fruiting by heterothallic isolates, attempts were made to fruit homothallic isolates of *A. mellea* from Japan, China and Africa (Table 5) using the optimal conditions identified for heterothallic isolate ELDO17. After four weeks colonisation of RST medium at room temperature, the cultures were moved into the first condition of warm / bright conditions of 23ºC, 125 µmol m^{-2} s^{-1} light, 16 h light / 8 h dark for six weeks, followed by cool / dim conditions of 15ºC, 5 µmol m^{-2} s^{-1} light for 10 h light / 14 h dark awaiting primordia emergence. Negative controls were cultures maintained in constant conditions of 15ºC, 5 µmol m^{-2} s^{-1} light, 10 h light / 14 h dark.

Fruiting bodies were produced from all isolates from Africa (03468/T1, 03469/T1 and 03470/T1) and the Japanese isolate (03384/2) (Figure 1), but not from the Chinese isolates (03332/2, 03345/1 and 03371/1). In contrast to heterothallic *A. mellea*, all African and Japanese isolates produced fruiting bodies when maintained in control conditions without a combined light and temperature reduction. Monosporous progeny of homothallic isolates had a flat, crustose morphology typical of a diploid culture.

3.5 Timing of primordia emergence

The timing of primordia emergence was recorded throughout the experiments and was found to vary between isolates. Primordia of ELDO17 were usually produced 23 days after a light and temperature reduction and 25 days after across all heterothallic cultures on average, whereas primordia induction in homothallic cultures generally took longer and was more variable (38 days). The number of days taken for primordia to appear in heterothallic cultures after a light and temperature reduction and the number of days after being moved to the growth rooms that primordia appeared in homothallic cultures (since primordia production was not dependent on a light or temperature reduction in these isolates) is shown in Figure 4.

Fruiting bodies of homothallic cultures developed more quickly (two weeks) than those of heterothallic cultures (three weeks) and the minimum time taken from inoculation of medium to harvesting basidiospores was 97 days for homothallic cultures and 107 days for heterothallic cultures. In some cases, two 'flushes' of homothallic *A. mellea* fruiting bodies could be obtained from one culture without altering the growth conditions, whereas new primordia that developed in cultures of heterothallic *A. mellea* after the first flush of fruiting bodies did not develop further.

3.6 Evaluation of hygromycin resistance vectors

Once a robust system for producing fruiting bodies *in vitro* was established, basidiospores were readily available for *A. tumefaciens*-mediated transformation. Transformation of basidiospores to

hygromycin resistance has been reported previously using *A. tumefaciens* AGL-1 and plasmid pBGgHg (Baumgartner *et al.* 2010) and this plasmid was used to transform *A. mellea* in addition to another plasmid previously utilised to transform basidiomycetes, pGR4-4iGM3 (Burns *et al.* 2005), and two plasmids constructed in this research, pCAM-hph-Pcgpd and pCAM-hph-series, in order to investigate transformation efficiency of *A. tumefaciens* strains and vectors. Transformations were performed at least twice for each strain and vector combination and the transformation efficiency was evaluated for *A. tumefaciens* strains AGL-1 and LBA1126 with the four plasmids. *Agrobacterium tumefaciens* strain LBA1126 was able to transform *A. mellea* with a similar efficiency to AGL-1, producing four and three transformants per transformation plate, respectively. The *gpd* promoter from *P. chrysosporium* was capable of driving *hph* expression and yielded a similar number of transformants to plasmids with the *A. bisporus* promoter driving *hph* (generating three and four transformants per transformation plate, respectively). PCR analysis showed the presence of the *hph* gene in all transformants tested and serial transfers on to PDA with 30 µg/ml hygromycin resulted in no loss of hygromycin resistance. Three colonies transformed with each plasmid were selected and subcultured in triplicate on to PDA with 30 µg/ml hygromycin to evaluate efficacy of transgene expression. Colonies transformed with a plasmid containing the full length *hph* gene demonstrated significantly faster growth after three weeks (according to Tukey's HSD test following one-way ANOVA), indicated by a larger colony diameter in comparison to colonies transformed with the truncated *hph* gene (Figure 5).

3.7 Fruiting body production in transgenic isolates

Fruiting was attempted in RST medium (Table 5) with selected transgenic isolates of *A. mellea* with diploid morphology produced in this study and transgenic isolates produced in previous work (Baumgartner *et al.* 2010) to assess stability of the transgene.

Isolates produced by pairing six different haploid transgenic isolates generated during transformation with plasmid pBGgHg of basidiospores from wild-harvested isolate Son202 with diploid wild-type NAPA187 that are believed to be triploid or aneuploid according to microsatellite data (Napa187xR1PT2 to Napa187xR2PT3) (Baumgartner *et al.* 2010) did not fruit. In contrast, transformants with either pBGgHg or pCAM-hph-series, which were generated from basidiospores from *in vitro*-produced fruiting bodies of ELDO17 and were diploid based on morphology (pB1 to 16.1), produced primordia 26 days after a light and temperature reduction. Primordia developed into immature fruiting bodies and mycelial cultures established from the stipe, pileus and gill tissue of the transgenic fruiting bodies maintained hygromycin resistance, verified by serial subcultures on to PDA with 30 µg/ml hygromycin.

4. Discussion

4.1 *In vitro* production of fruiting bodies

An effective method of producing *in vitro* fruiting bodies has been established for heterothallic *A. mellea* from the western US. Previously recalcitrant to fruiting *in vitro*, this protocol for fruiting *A. mellea* is reliable and repeatable and has produced mature fruiting bodies of two heterothallic western US isolates, ELDO17 and ELDO19, four homothallic isolates (03468/T1, 03469/T1, 03470/T1 and 03384/2) and immature fruiting bodies of a heterothallic UK isolate, CG440. A medium consisting of rice, sawdust and tomato (RST) was shown to be the most effective, and of the heterothallic isolates, ELDO17 fruited most reliably. The optimal parameters for *in vitro* fruiting in heterothallic isolate ELDO17 were determined to be the following: RST medium colonisation at room temperature for four weeks followed by warm / bright conditions of 23ºC, 125 µmol m^{-2} s^{-1} light, 16 h light / 8 h dark for six weeks, followed by cool / dim conditions of 15ºC, 5 µmol m^{-2} s^{-1} light, 10 h light / 14 h dark. Primordia appeared within three to four weeks after a light and temperature decrease and fruiting bodies matured within an additional three to four weeks. Primordia were only induced three to four weeks after a temperature reduction in cultures exposed to light and this was most successful with a simultaneous decrease in light intensity. Mature fruiting bodies with viable basidiospores were obtained in less than four months at any time of year.

Fruiting body development in basidiomycetes is usually induced by a substantial change in environmental conditions, often light and temperature. In several species such as *Pleurotus ostreatus*, *Schizophyllum commune, Lentinula edodes* and *Flammulina velutipes*, fruiting is controlled as least partially by light, which has been shown to influence hyphal aggregation required for primordia formation and affect maturation of the basidiocarp (Arjona *et al.* 2009; Kües & Liu, 2000; Nakazawa *et al.* 2008). In other species such as *A. bisporus*, fruiting is not light-regulated and is dependent upon a temperature reduction of at least 5°C. *Coprinopsis cinerea*, *L. edodes* and *F. velutipes* also require a temperature reduction in order to induce fruiting (Eastwood *et al.* 2013; Kües & Liu, 2000; Nakazawa *et al.* 2008).

A requirement for a temperature reduction to induce primordia has been reported previously in *Armillaria*, in an undescribed species from Africa, where cultures colonised at 25°C required a temperature decrease to 20°C to initiate primordia development (Otieno *et al.* 2003). Fruiting in other *Armillaria* species, however, does not appear to be reliant upon a temperature reduction, or perhaps the higher colonisation temperature prior to movement into cooler growth rooms provides a sufficient temperature decrease. For example, the majority of protocols for fruiting other *Armillaria* species stipulate colonisation in the dark between 20-27°C and then incubation in 12 h light / 12 h dark at 12-25°C awaiting primordia emergence (Grillo *et al.* 2000; Ota *et al.* 1998; Otieno *et al.* 2003; Pérez-Sierra *et al.* 2004; Reaves & McWilliams, 1991; Shim *et al.* 2006); sometimes providing a temperature reduction between colonisation and incubation. Other protocols have maintained cultures in constant light at 20-23°C during colonisation and incubation (Shaw *et al.* 1981) or colonised the medium at 23°C and incubated cultures in the laboratory without controlling light and temperature (Abomo-Ndongo *et al.* 1997) so that a temperature decrease does not occur. Furthermore, with the exception

of cultures maintained on a laboratory bench that may have experienced a decrease in day length, none of these protocols used by other researchers appears to have provided a light reduction, since colonisation occurred in the dark. As this research has shown that primordia induction in heterothallic *A. mellea* requires a temperature decrease, and colonisation at a higher temperature prior to transfer into a cooler growth room provides an insufficient temperature decrease to induce primordia unless a light reduction is also provided, any attempted fruiting of heterothallic *A. mellea* using these conditions is unlikely to have succeeded.

In correlation with previous studies, homothallic isolates of *A. mellea* from Japan, and from Africa (which are intercompatible, likely to have originated from Japan and one of which has been fruited previously (Abomo-Ndongo *et al.* 1997; Baumgartner *et al.* 2012), fruited with no light or temperature reduction or with only a reduction in temperature between colonisation and incubation. The reasons for this remain elusive but similar fruiting conditions have been described for other *Armillaria* species (Grillo *et al.* 2000; Reaves & McWilliams, 1991; Shim *et al*. 2006) and are possibly reflective of the seasonal conditions of their natural habitats. The homothallic isolates of *A. mellea* from China (CBS G), heterothallic *A. mellea* isolate NAPA187 and *A. gallica* isolates ANA220, CG024 and CG263 did not fruit in this study, and heterothallic *A. mellea* isolate CG440 and *A. gallica* isolate CG645 only produced immature fruiting bodies and primordia, respectively. It is possible that a longer duration of warm / bright conditions or a sharper temperature decrease would induce primordia in these isolates, or different medium may be more effective, since fruiting body production in ELDO17 and ELDO19 varied according to medium and *A. gallica* has been fruited on RSP previously (Grillo *et al.* 2000; Reaves & McWilliams, 1991) but this medium was only attempted with one *A. gallica* isolate in this study. Alternatively, these isolates may not be fully fertile and so unable to produce mature fruiting bodies irrespective of the conditions.

4.2 Evaluation of *Agrobacterium tumefaciens* transformation vectors and transgenic isolates

The production of *A. mellea* fruiting bodies *in vitro* facilitates research requiring regular availability of basidiospores. In this study, we have shown that *in vitro* produced basidiospores from isolate ELDO17 can be transformed by *A. tumefaciens* strains LBA1126 and AGL-1 with similar efficiencies to produce hygromycin resistant colonies, using both the full length and truncated *hph* gene, and verified that the *P. chrysosporium gpd* promoter is able to drive *hph*. This is significant because the truncated *hph* gene is unable to confer hygromycin resistance in some basidiomycetes such as *C. cinerea* and *Clitopilus passeckerianus* (Kilaru *et al.* 2009), yet we have shown that it can confer hygromycin resistance in *A. mellea*, albeit at lower efficiencies than the full length version of the gene. This decrease in hygromycin resistance with vectors that contain the truncated version of the *hph* gene, demonstrated by slower growth of transformed colonies, is attributed to the deletion of two lysine residues at the 5' end of the *hph* gene, reducing the efficacy of hygromycin deactivation. As certain promoters are unable to drive *hph* in *A. bisporus* and *C. cinerea* (Burns *et al.* 2005, Burns *et al.* 2006), the ability of the *P. chrysosporium gpd* promoter to drive *hph* in *A. mellea* is also important and will increase the availability of functional promoters for genetic work in *A. mellea*. Furthermore,

the construction of plasmid pCAM-hph-series, demonstrating similar transformation efficiencies to the commonly used basidiomycete vector pBGgHg and simplifying promoter and marker gene exchange by the use of the yeast homologous recombination system, will facilitate future genetic studies in *A. mellea*.

The transgenic isolates derived from ELDO17 generated in this work could be induced to produce immature fruiting bodies, and mycelial cultures established from fruiting body tissues maintained hygromycin resistance. Growth arrested in transgenic fruiting bodies a few days before maturity, in a similar way to that observed in wild-type isolates on occasion, and no basidiospores were obtained. Since ELDO17 is heterothallic and sibling mating between monosporous progeny during co-incubation of the basidiospores and *A. tumefaciens* probably gave rise to the diploid mycelium, it is probable the genetically variable progeny will have variable, and possibly reduced, fertility, so the maturation of fruiting bodies is more likely in some progeny than in others. Isolates that were produced via pairing of haploid transgenic isolates derived from Son202 with wild-type diploid isolate NAPA187 (Baumgartner *et al.* 2010) that are believed to be triploid or aneuploid did not produce primordia, possibly due to the their ploidy status leading to complications in fruiting, or because *in vitro* fruiting was not successful in parent isolate NAPA187 and therefore is less likely to succeed in its progeny, and *in vitro* fruiting has not been attempted in parent isolate Son202 and thus its fruiting ability is unknown.

4.3 Conclusions

The ability to fruit heterothallic *A. mellea in vitro* will assist future research by reliably providing basidiospores of a known genotype for use in transformation studies, ending reliance on sporadically available wild-collected basidiospores. The knowledge of the mechanisms controlling *in vitro* production of *A. mellea* fruiting bodies may also assist with fruiting of other *Armillaria* species that are difficult to fruit in culture. Furthermore, the evaluation of suitable *A. tumefaciens* strains for transformation, and the construction of a versatile vector, should facilitate future genetic research investigating fluorescent protein expression and gene silencing in this important pathogenic species.

5. Acknowledgements

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6. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Table 1 – Isolates of *Armillaria gallica* and *A. mellea* used in this study				
Taxon	**Isolate**	**Location**	**Host**	**Collector(s) / Reference / Year**
A. gallica (heterothallic)	ANA220	UK	*Acer* sp.	RHS, 2003
	CG024	Surrey, UK	*Acer* sp.	RHS, 2004
	CG263	Yorkshire, UK	Rhizomorphs	RHS, 2006
	CG645	North Ayrshire, UK	*Acer* sp.	RHS, 2007
A. mellea (heterothallic)	CG440	Surrey, UK	*Ligustrum* sp.	RHS, 2006
	NAPA187	California, USA	*Quercus kelloggii*	K. Baumgartner, 2007
	ELDO17	California, USA	*Vitis* sp.	K. Baumgartner, 2000
	ELDO19	California, USA	*Vitis* sp.	K. Baumgartner, 2000
A. mellea ssp. *africana* (homothallic)	03468/T1 [ST1]	Sao Tome, Africa	*Theobroma cacao*	M. Ivory
	03469/T1 [K5]	Kenya, Africa	*Cupressus funebris*	I.A.S. Gibson, 1958
	03470/T1 [K8]	Kenya, Africa	*Grevillea robusta*	M. Ivory
Chinese Biological Species G (homothallic)	03332/2	Yunnan province, China	*Rhododendron* sp.	G-F. Qin, J. Zhao, 2003
	03345/1	Hubei province, China	*Ailanthus altissima*	J. Zhao, 2003
	03371/1	Guangxi province, China	Broadleaf tree	J. Zhao, 2003
A. mellea ssp. *nipponica* (homothallic)	03384/2	Japan	Unknown	G-F. Qin, 2003
Armillaria mellea (heterothallic, transgenic)	Napa187xR1PT2	n/a	n/a	Baumgartner *et al.* (2010)
	Napa187xR1PT4			
	Napa187xR1PT5			
	Napa187xR1PT6			
	Napa187xR2PT2			
	Napa187xR2PT3			
	pB1	n/a	n/a	This paper
	pM1			
	15.2			
	16.1			

Table 2 – Vectors used in transformations				
Plasmid	**Promoter**	**Hygromycin resistance gene**	**Terminator**	**Reference**
pBGgHg	*Agaricus bisporus gpdII*	Full length	CaMV 35S	Chen *et al.* (2000)
pGR4-4iGM3	*Agaricus bisporus gpdII*	Truncated	*Aspergillus nidulans trpC*	Burns *et al.* (2005)
pCAM-hph-Pcgpd	*Phanerochaete chrysosporium gpd*	Truncated	*Aspergillus nidulans trpC*	This paper
pCAM-hph-series	*Agaricus bisporus gpdII*	Full length	CaMV 35S	This paper

Table 3 – Fruiting of isolates in preliminary studies						
Species	**Isolate**	**Medium**				
		Oranges	Rice, sawdust and peptone (RSP)	Rice, sawdust and carrot (RSC)	Rice, sawdust and tomato (RST)	Rice, sawdust pearl barley, corn and carrot (RSBCC)
A. gallica	ANA220	0% [a]	0% [c]	0% [b]	0% [e]	n/a
	CG024	0% [a]	n/a	0% [a]	0% [e]	n/a
	CG263	0% [a]	n/a	0% [a]	0% [e]	n/a
	CG645	0% [a]	n/a	100% primordia [a]	25% primordia [e]	n/a
A. mellea	CG440	0% [b]	0% [a]	0% [a]	50% immature fruiting bodies [e]	n/a
	ELDO17	0% [a]	0% [a]	100% immature fruiting bodies [a]	33% mature fruiting bodies [d]	33% Immature fruiting bodies [d]
	ELDO19	0% [a]	0% [b]	0% [a]	100% immature fruiting bodies [a]	n/a
	NAPA187	0% [a]	n/a	0% [b]	0% [e]	n/a

Where superscript **= a** n=1, experiment not repeated; **b** n=2, experiment repeated once; **c** n=3, experiment not repeated; **d** n=3, experiment repeated once; **e** n=4, experiment repeated once

Table 4 – Conditions used for light and temperature reduction experiment for fruiting body production in ELDO17 on RST medium			
Condition	**First condition**	**Second condition**	**Temperature or light reduction**
1	23ºC, 125 µmol m^{-2} s^{-1}	n/a	Constant warm/bright; no light or temperature reduction
2	23ºC, 5 µmol m^{-2} s^{-1}	n/a	Constant warm/dim; no light or temperature reduction
3	23ºC, dark	n/a	Constant warm/dark; no light or temperature reduction
4	23ºC, 125 µmol m^{-2} s^{-1}	23ºC, 5 µmol m^{-2} s^{-1}	Warm/bright to warm/dim; light reduction
5	23ºC, 125 µmol m^{-2} s^{-1}	15ºC, 125 µmol m^{-2} s^{-1}	Warm/bright to cool/bright; temperature reduction
6	23ºC, 125 µmol m^{-2} s^{-1}	15ºC, 5 µmol m^{-2} s^{-1}	Warm/bright to cool/dim; light and temperature reduction
7	23ºC, 5 µmol m^{-2} s^{-1}	15ºC, 5 µmol m^{-2} s^{-1}	Warm/dim to cool/dim; temperature reduction
8	23ºC, dark	15ºC, dark	Warm/dark to cool/dark; temperature reduction
9	[initially colonised at RT] 15ºC, 125 µmol m^{-2} s^{-1}	15ºC, 5 µmol m^{-2} s^{-1}	Cool/bright to cool/dim; light reduction
10	[initially colonised at 15ºC] 15ºC, 125 µmol m^{-2} s^{-1}	15ºC, 5 µmol m^{-2} s^{-1}	Cool/bright to cool/dim; light reduction
11	15ºC, 125 µmol m^{-2} s^{-1}	n/a	Constant cool/bright; no light or temperature reduction
12	15ºC, 5 µmol m^{-2} s^{-1}	n/a	Constant cool/dim; no light or temperature reduction
13	15ºC, dark	n/a	Constant cool/dark; no light or temperature reduction

Table 5 – Fruiting of homothallic and transgenic isolates in RST medium		
Species	**Isolate**	**Fruiting bodies produced**
Chinese Biological Species G (homothallic)	03332/2	No primordia produced [b]
	03345/1	No primordia produced [a]
	03371/1	No primordia produced [a]
A. mellea ssp. *nipponica* (homothallic)	03384/2	100% Mature fruiting bodies [b]
A. mellea ssp. *africana* (homothallic)	03468/T1	75% Mature fruiting bodies [a]
	03469/T1	75% Mature fruiting bodies [a]
	03470/T1	83% Mature fruiting bodies [b]
Isolates generated from pairing diploid WT NAPA187 with haploid transgenic isolates derived from Son202	Napa187xR1PT2	No primordia produced [b]
	Napa187xR1PT4	No primordia produced [b]
	Napa187xR1PT5	No primordia produced [a]
	Napa187xR1PT6	No primordia produced [a]
	Napa187xR2PT2	No primordia produced [a]
	Napa187xR2PT3	No primordia produced [a]
Transgenic isolates	pB1	33% Immature fruiting bodies [c]
	pM1	100% Immature fruiting bodies [c]
	15.2	100% Immature fruiting bodies [d]
	16.1	100% Immature fruiting bodies [d]

a n=4, experiment not repeated; **b** n=6, experiment repeated once; **c** n=3, experiment not repeated; **d** n=2, experiment not repeated;

Figure 1: *In vitro* fruiting bodies produced on RST medium. **A** Clusters of heterothallic ELDO17 primordia and small immature fruiting bodies; **B** Mature ELDO17 fruiting bodies; **C** Mature harvested fruiting bodies of ELDO17; **D** Mature fruiting bodies of homothallic African isolate 03470/T1; **E** Mature fruiting bodies of homothallic Japanese isolate 03384/2

Figure 2: The percentage of heterothallic *A. mellea* ELDO17 and ELDO19 cultures that produced primordia (A) and mature fruiting bodies (B) on various media following a combined light and temperature reduction.

Error bars represent standard error from the mean. RSP = rice, sawdust and peptone medium; RSC = rice, sawdust and carrot medium; RST = rice, sawdust and tomato medium

Figure 3: Percentage of heterothallic *A. mellea* ELDO17 cultures that produce mature fruiting bodies under different light and temperature regimes on RST medium. n = 3 to 6.

Figure 4: Days until primordia emergence on RST medium for different *A. mellea* isolates after movement into second growth room.

Error bars represent standard error from the mean.

Figure 5: Average colony diameter of selected transformants with different plasmids grown on PDA + 30 μg/ml hygromycin after three weeks.

n = 9 and error bars represent standard error from the mean. Letters above bars indicate significant differences between groups as determined by Tukey's HSD test following one-way ANOVA where P = <0.05.

Supplementary Figure 1 – pCAMBIA0380 adapted to function in yeast to construct vectors pCAM-hph-Pcgpd and pCAM-hph-series

Figure 1

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Figure 2

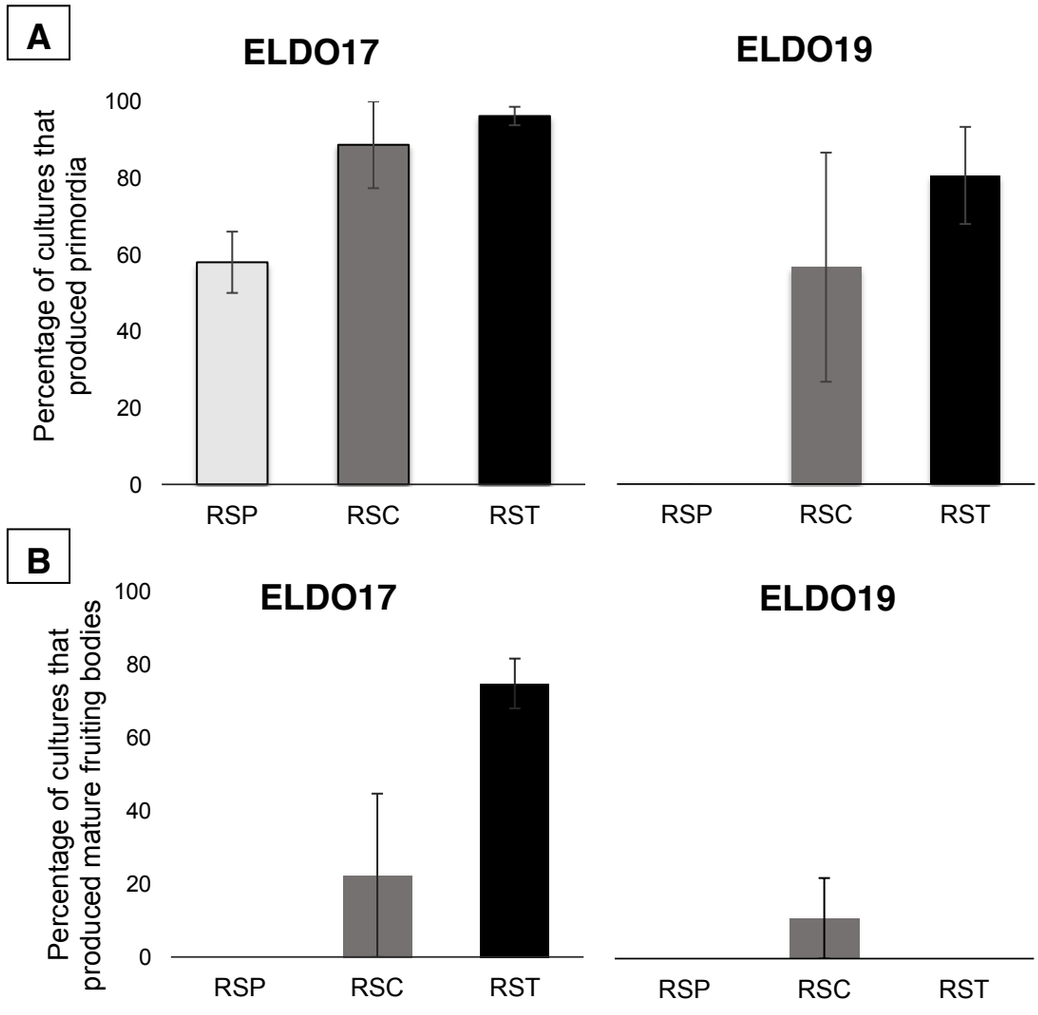

A
ELDO17
ELDO19
Percentage of cultures that produced primordia
100
80
60
40
20
0
RSP
RSC
RST
B
ELDO17
ELDO19
Percentage of cultures that produced mature fruiting bodies
100
80
60
40
20
0
RSP
RSC
RST

Figure 3

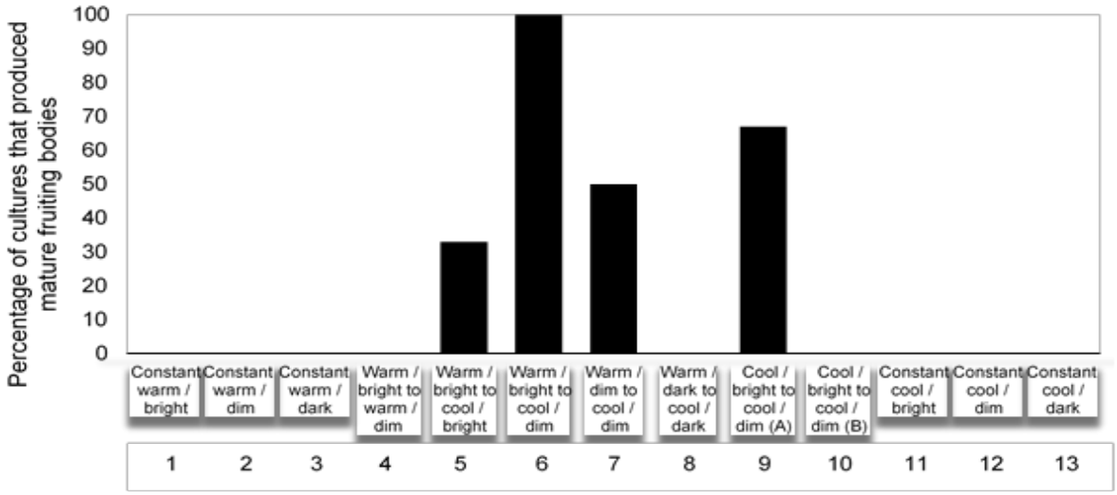

Percentage of cultures that produced mature fruiting bodies
100
90
80
70
60
50
40
30
20
10
0
Constant warm / bright
Constant warm / dim
Constant warm / dark
Warm / bright to warm / dim
Warm / bright to cool / bright
Warm / bright to cool / dim
Warm / dim to cool / dim
Warm / dark to cool / dark
Cool / bright to cool / dim (A)
Cool / bright to cool / dim (B)
Constant cool / bright
Constant cool / dim
Constant cool / dark
1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 4

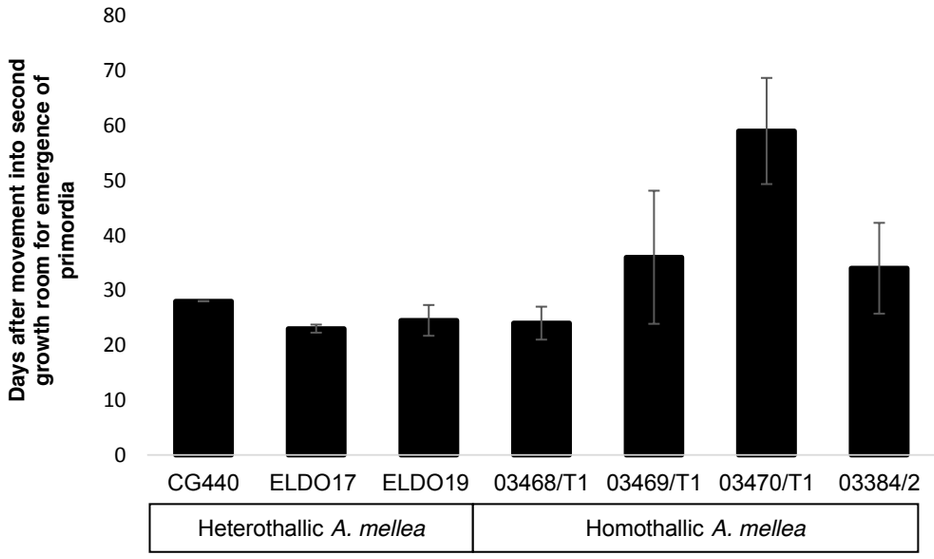
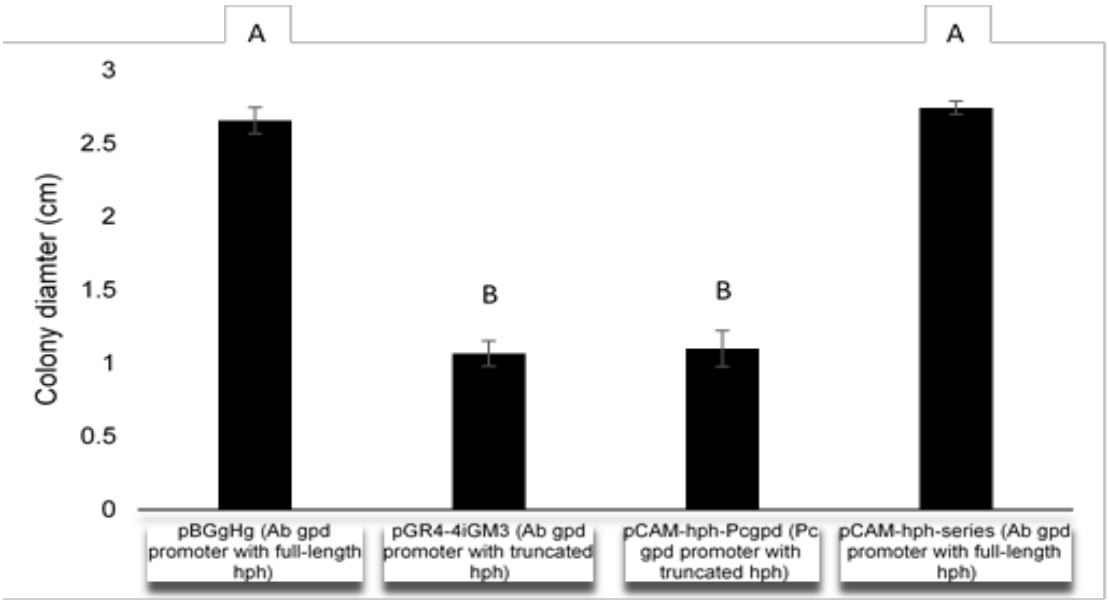

Days after movement into second growth room for emergence of primordia
80
70
60
50
40
30
20
10
0
CG440
ELDO17
ELDO19
03468/T1
03469/T1
03470/T1
03384/2
Heterothallic A. mellea
Homothallic A. mellea

Figure 5


A
A
B
B
Colony diamter (cm)
3
2.5
2
1.5
1
0.5
0
pBGgHg (Ab gpd promoter with full-length hph)
pGR4-4iGM3 (Ab gpd promoter with truncated hph)
pCAM-hph-Pcgpd (Pc gpd promoter with truncated hph)
pCAM-hph-series (Ab gpd promoter with full-length hph)

Supplementary Table 1

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Supplementary Figure 1

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